Author's response to reviews

**Title:** CD44 isoforms are heterogeneously expressed in breast cancer and correlate with tumor subtypes and cancer stem cell markers

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**Author's response to reviews:** see over
Dear Editor,

Thank you for the review comments on our manuscript MS: 2286430625502399 entitled "CD44 isoforms are heterogeneously expressed in breast cancer and correlate with tumor subtypes and cancer stem cell markers". We are pleased that the reviewers found the manuscript interesting and we have aimed to address the issues and questions raised.

We have answered the reviewers’ points one by one below and the changes in the manuscript have been highlighted.

We hope you will now find the manuscript suitable for publication in BMC Cancer and look forward to your decision.

Best regards,

Eleonor Olsson

Response to Reviewers

Reviewer 1

Minor Essential revision:
1- In the first paragraph of results, authors conclude that the concordance of CD44 expression (determined by Flow Cytometry) and mRNA expression (determined by qRT-PCR) is very good. However, in table1, Flow Cytometry results are expressed as % of positive cells instead of MFI. Also, some results are not in concordance since 79.6% MDA-MB-361 are positive for CD44 expression and the relative mRNA expression is 0.95 while 65.8% of HCC1428 are CD44+ with a relative mRNA expression of 8.96. Authors have to clarify this point.

We have presented the flow cytometry results as % of positive cells and not as MFI since the former is most frequently used in earlier publications on this topic (Sheridan et al., 2010, Breast Cancer Res 8(5): R59, Hwang-Verslues et al, 2009, PloS one 4(12): e8377). Of practical reasons the FACS data were obtained at different time points for the different cell lines. Since we were using slightly different settings from time to time this means that MFI values are difficult to compare.

Our result with a high percentage of CD44+ cells for the MDA-MB-361 cell line is in agreement with earlier studies (Sheridan et al., 2010, Hwang-Verslues et al., 2009). The antibody for CD44 binds to an extracellular epitope of the protein, but for the gene expression assay of total CD44, a probe corresponding to a region
coding for parts of the cytoplasmic tail of the molecule was analyzed. Very recently, a variant of CD44 was discovered that lacks the sequence we use for detection of total CD44 by q-RT-PCR, which possibly could explain our results.

More likely the relatively high amount of synthesized CD44 protein in MDA-MB-361 can be explained by for example post-transcriptional modifications and translational regulation specific only for this cell line.

This has been clarified in the manuscript by adding the following sentences in the Results section: “However, the MDA-MB-361 cell line has 79.6% of CD44 positive cells despite a moderate expression level of total CD44 (Table 1). Possibly, the relatively high amount of synthesized CD44 protein in MDA-MB-361 can be explained by for example post-transcriptional modifications and translational regulation specific only for this cell line.”

2- Figure 2B illustrate the CD44 variants appearance under non-adherent conditions in JIMT-1. Did you perform the same experiment using the other cell lines? If yes, please clearly detail it in the text.

We do not have Western data available for the other cell lines. We selected JIMT-1 since the Western worked best (resulting in strong bands) for that cell line. We had problems with low signals using the other cell lines.

3- Authors must justify why they focused on the 3 isoforms described in this study, in addition of CD44s.

At the time we initiated the study only the isoforms CD44v2-v10 (NM_000610.3), CD44v3-v10 (NM_001001389.1), CD44v8-v10 (NM_001001390.1), CD44S (NM_001001391.1) and CD44RC (NM_001001392.1) were accepted as Reference sequences in the National Center for Biotechnology Information (NCBI) database. In our study, the transcript NM_001001392.1 had extremely low expression levels and was hence excluded from the study. Recently, three new sequences NM_001202555.1, NM_001202556.1, NM_001202557.1 have been added to the NCBI database, but unfortunately we have no possibility to include these newly registered transcripts in this study.

The information described above has been included in the Material and Methods section in the paragraph “Quantitative real-time PCR”.

Discretionary revisions:
1- Paragraph titles can be improved by making sentences that summarize the data presented in each paragraph.

We have changed a number of paragraph titles in the Results section to make them more explicit.

Reviewer 2
Has a quality control of cell lines been performed?

*The cell lines have been subjected to aCGH analysis to ensure that no contamination of cell lines had occurred.*

Flow cytometry analysis of CD44 and CD24 should be done on mammospheres.

*The results of these experiments have been added to the Results section in the paragraph “Altered expression of CD44 isoforms in mammosphere cultures”.*

Aldefluor assay might be carried out in addition to ALDH1 protein expression since, for CSCs, enzyme activity is more relevant than protein expression.

*Unfortunately it is not possible to perform the Aldefluor assay on the tumor material since we only have access to snap-frozen or paraffin-embedded tissue. As Aldeflour measures the enzyme activity, viable cells are needed for the assay. ALDH1A1 immunostaining has in several previous publications been used as a substitute in large tumor materials, although not completely overlapping.*

Results

It is stated that the concordance between CD44 mRNA and protein expression was very good in breast cell lines. It is not obvious that data shown in table 1 support this conclusion which is not really a scientific statement. What means a very good concordance?

*To clarify, the statement mentioned above has been changed in the manuscript to: “The total expression of CD44 transcripts was first compared to flow cytometry data of CD44 and a relatively good agreement between mRNA and protein expression could be seen.”*  
See also response number 1 to Reviewer 1.

Discussion

Does previous data clearly demonstrate that CD44 is a target of gene amplification?

*CD44 has been shown to be amplified in breast cancer (Klingbeil et al., Breast Cancer Res Treat, 2010, 120:95-109). However, to clarify, we have changed the sentence in the Discussion section to “Nevertheless, since CD44 has been shown to be amplified and overexpressed in breast cancer, this implicates a functional role in tumor development and growth”.*